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Codon Usage Analysis of Aminopeptidase N Gene in Order Diptera

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Abstract

Aminopeptidase N (APN) has been identified as Cry toxin-binding proteins in various insects and also responsible for digestion of peptides. An attempt has been made on the APN orthologous to overview the genome-wide analysis of APN gene in Order Diptera. The entire ORF of APN gene sequences belonging to mosquito and non-mosquito species in Order Diptera have been retrieved from NCBI and analysed by various bioinformatics tools. The goal of this study is to perform comparative analysis of APN nucleotide sequences, nucleotide composition, codon usage bias pattern, amino acid composition bias, pattern of conservation among amino acid residues, and phylogenetic analysis among insect species belonging to the order Diptera. Our results may provide useful insights on the patterns of codon usage bias that facilitate a better understanding of the structure and evolution of gene coding sequences of these insects.

Keywords: *Anopheles gambiae*, *Aminopeptidase N*, *Paralogous gene*, *Genome*, *Multigene*, *Evolution*, *Aedes aegypti*, *APN genes*, *Malaria*, *Dengue*.

Introduction

Mosquito-borne diseases continues to remain a life threatening infectious disease throughout the tropical region of the world. The global health and socioeconomic burden imposed by the mosquitoes is well established, with an estimated 282 million malaria cases and 14 million deaths reported worldwide in 2024 (WHO Report, 2025). It is a leading infectious disease of humans worldwide. It kills around 610 000 deaths over the world every year that account for a large number of death cases worldwide. In 2024, more than 14.6 million dengue cases were reported to WHO, with over 9,000 dengue-related deaths globally. The midgut is the site where the parasite first interacts with mosquito tissue and further developmental process takes place. The mosquito's midgut represents one of the most challenging environments for survival of parasites and thus midgut is a striking site for novel target malaria control strategies. The work reported suggests that genetic modification of mosquito vectorial capacity is feasible and represents a major step toward the goal of containing the spread of malaria. Strong and ubiquitous promoters of midgut could be used to drive the expression of effector genes that hinders transmission by either killing or interfering with parasite development. The alanyl Aminopeptidase N is the leading malarial TBV immunogen [1]. A midgut-specific protein, Aminopeptidase N 1 (APN1) is glycosylphosphatidylinositol anchored protein reported to play an important role in ookinete invasion of Plasmodium in the *An. gambiae*. The Anopheline Alanyl APN is originally isolated from the apical brush border microvilli fraction of *An. gambiae* midguts [2]. It is a ubiquitous enzyme which is found in a wide range of organisms from insects to mammals. APN belongs to a group of membrane-bound zinc enzymes [3] Dipteran Aminopeptidase N genes were observed to be highly biased genes among all the orders of class Insecta as revealed through nucleotide composition. In this study, an attempt has been made to overview genome-wide identification of APN genes in Diptera. The study covered comparative analysis of APN nucleotide sequences, nucleotide composition, codon usage bias pattern, amino acid composition analysis among insects to better understand the evolution of APN and its biological significance. A comparative sequence analysis was carried out to gain better understanding of the evolution of APN gene. The analysis of complete gene and CDS sequences of Aminopeptidase N was performed. The ORF of APNs belonging to Diptera order of class Insecta have been retrieved from various databases such as NCBI, Uniprot KB, vectorbase and flybase database etc and have been analysed by applying various bioinformat-

ics tools. All the sequences show significant similarity with each other as revealed after performing BLAST. Although mosquitoes APN genes have diverged considerably from one another, the complete sequence can be aligned along their entire length. The nucleotide composition analysis was performed using Genomics GC% Content Calculate. The Amino acids composition of all known Aminopeptidase N proteins revealed mosquito-specific amino acid usage. The extent of codon bias in mosquito Aminopeptidase N genes based on the Relative Synonymous Codon Usage values was also determined. Gene structure study shows high sequence variations among them. In addition, nucleotide sequence alignment, intron analysis and nucleotide composition was carried out. The genus wise multiple sequence alignment of APN gene was performed using CLUSTAL W. Sequence comparisons of the genes showed that, except in conserved regions, there are many nonsynonymous substitutions, suggesting that the APN genes are highly evolving genes. Phylogenetic analyses were also carried out to establish the phylogenetic relationships among different orthologs present in other organisms. Protein-protein interactions show that APN is highly connected protein, supporting their role as hub with glutathione metabolism, Glutathione acts as insecticide resistance, so disrupting of APN protein or any other protein, this protein network may block the whole metabolic pathway of Glutathione. Models of proteins were also studied to further expand the knowledge of functioning of the gene.

MATERIALS AND METHODS

Retrieval and analysis of Mosquitoes APN1 orthologous gene sequences: The APN1 gene sequences of 15 different mosquitoes and 15 non mosquitoes have been retrieved from Vectorbase, Flybase, UniProt KB and NCBI databases for comparison. By using these databases, the functional annotation of genes, number of nucleotides, gene products, gene orientation and position, chromosome mapping and amino acids sequences were analysed in all orthologous.

Sequence analysis: BLAST searches were carried out to find sequences with similarity in the databases. Sequences for alignment and phylogenetic analysis were retrieved from the Genbank. Multiple sequence alignments were performed using the Bioedit tool. MEGA 7 software was used to analyse and establish phylogenetic relationships among the mosquitoes APN1 genes [4].

Conserved domains identification for APN proteins: The conserved domains were identified in all orthologous APN sequences by various domain search tools

like NCBI CDD (Conserved domain database) [5], Pfam [6] and InterProScan [7] software, which in turn by searching significantly close orthologous family members. The predicted cleavage sites signal peptides were analysed by using Signal IP algorithm. Glycosylation and phosphorylation sites were predicted by using the Prosite tool [8]. Possible transmembrane domains in APN proteins were also found by using the TMHMM [9]. The Molecular Weight (MW) and Isoelectric point (pI) were calculated by Compute pI/MW [10]. Multiple sequence alignments were performed with the BIOEDIT program with all paralogous sequences.

Nucleotide Composition Analysis: General nucleotide composition (A, T, C and G %) and nucleotide composition at first, second and third position of each codon were analysed for dipteran insect Aminopeptidase N CDS using MEGA6 and Genomics % GC content calculator. The AT3 and GC3 indices refer to the third position of synonymous codon and it helps to depict the biasness of the gene [11].

Synonymous Codon Usage: Relative synonymous codon usage (RSCU) is calculated as the ratio of the observed frequency of a codon to the frequency expected if all synonymous codons of a particular amino acid are used equally [12]. RSCU values were analysed using MEGA and Calcal software. The effective number of codons used by a gene (ENC) is generally used to measure the bias of synonymous codons [13]. Codon Adaptive Index (CAI) is a measure of relative adaptiveness of codon usage of a gene towards the codon usage of highly expressed genes. Calcal software was used to calculate ENC and CAI values [14].

Multiple sequence alignment and Phylogenetic analysis: Order wise multiple sequence alignment was performed with MEGA 6 and BIOEDIT programmes in all insect APN. This helps to depict conserved characteristic features among these insect species. Phylogenetic tree were generated by MEGA6 using Bootstrap consensus Neighbour-joining Method [15-16]. These trees were constructed using APN protein sequences to infer the evolutionary distances among insects.

RESULTS

Nucleotide Sequence Analysis: To understand the evolutionary changes of APN gene among Diptera of insects, the complete coding sequences of fifteen mosquitoes and non-mosquitoes from Diptera orders have been obtained from the NCBI/ Genbank and UniProtKB databases. Further, In silico comparative analysis was performed. All orthologous sequences were compared and analysed for conserved sites using software called MEGA6.06 [14]. The multiple sequence

alignments were carried out to detect the conserved sequences [17]. Dipteran APN from different classes shows divergence from one another, moreover, APN genes in mosquitoes reveal strong similarities to each other (Table 1 and 2). However, the nucleotide coding sequence was found to have different numbers and positions of exons (Table 1 and 2). Most of the mosquitoes APN gene possess almost similar ORF size, within a range of 3-4 kb except *Aedes aegypti*. AUG and TAA (mostly) are used as a start and stop codon, respectively. Nucleotide sequence alignments of dipteran APNs have shown the presence of limited conservation at few short coding regions.

Nucleotide Composition of APN: Nucleotide Composition analysis of mosquitoes APN coding regions shows that the Anopheles APN genes were found to have higher GC content as compared to *Aedes* and *Culex* APN genes. The analysis shows GC content varies significantly among different species. Among Anopheles, *An. gambiae* has the highest GC content of (54.4%), while among *Aedes* and *Culex*, *Culex* has more GC (50.4%) content. The *Aedes* surprisingly has lower GC content, lower than 50% and measured with highest AT content of 51.5% among all mosquitoes. The data is shown Table 3 for Anopheles, *Aedes* and *Culex* respectively. The % GC and AT content of *An. gambiae* APN gene was found to be 56.1 and 43.9 respectively, while the values for GC1s, GC2s and GC3s are 55%, 43% and 70% respectively. The frequency of codons ending with G/C is higher than A/T in all mosquitoes. Table 3 shows the distribution of overall GC content of APN gene in various mosquitoes. The nucleotide composition analysis of non-mosquitoes was also performed and found that the APN gene belonging to *Drosophila* were found to be GC rich except some other species (Table 4). Dipterans APN gene was found to be GC rich and as compared to 1st and 2nd codon position high GC content was observed at 3rd codon position. This clearly indicates that the Diptera APN gene is a biased gene (Table 3 4). The overall nucleotide composition and composition at the third codon position in APN gene suggests that compositional constraint might be influencing the codon usage pattern of these species.

Amino Acids Composition: As APN proteins are reported to play an important role in digestion of blood. The deduced amino acids composition of insect APN proteins was determined. The amino acids composition of mosquito APN is shown in Table 5. Three amino acids residues; Alanine, leucine and threonine were found to be very rich in this protein, showing that these residues may be in high biological demand during digestion of blood. There was no other significant difference found in the amino acids composition of

Table 1. List of genomic sequences of APN gene of various mosquitos (Diptera).

S No	Species	Accession no.	Length (bp)	Start codon	Stop codon	ORF (bp)	Exon No.	AA	Mol. Wt (kDa)	PI	Signal peptide	Zn binding site	Peptidase_M1 domain	ERA_P1_C domain	Threonine rich region
1	Anopheles gambiae	AGAP004809	3588	ATG	TAA	3063	5	1020	113	5.0	1-19	H366E YAH 370	292-519	597-905	887-933
2	Anopheles arabiensis	AA016470	3586	ATG	TAA	3063	5	1020	113	5.1	1-19	H366E YAH 370	292-519	597-905	947-993
3	Anopheles merus	AM002547	3599	ATG	TAA	3063	5	1020	113	5.1	1-19	H366E YAH 370	292-519	597-905	947-993
4	Anopheles farauti	AF015666	3704	ATG	TAA	3078	5	1025	114	5.0	1-25	H366E YAH 370	292-519	597-904	949-998
5	Anopheles quadrianulatus	AQ016895	3586	ATG	TAA	3063	5	1020	113	5.0	1-19	H366E YAH 370	292-519	597-905	947-993
6	Anopheles sinensis	AS009153	3688	ATG	TAA	3093	5	1030	114	4.9	1-20	H365E LAH 369	291-518	596-902	907-1006
7	Anopheles atroparvus	AA011993	3872	ATG	TAA	3072	5	1025	113	4.9	1-20	H361E YAH 365	287-514	592-892	942-997
8	Anopheles darlingi	AD006959	3612	ATG	TAG	3060	5	1019	113	5.1	1-23	H360E YAH 364	286-513	591-894	939-1003
9	Anopheles maculatus	AM007648	3805	ATG	TGA	2775	5	924	104	5.0	-	H356E YAH 360	282-509	587-892	-
10	Anopheles albimanus	AA015678	3591	ATG	TAA	3048	5	1015	113	5.1	1-23	H359E YAH 363	285-512	590-893	889-999
11	Anopheles culicifacies	MK033514	3517	ATG	TAA	3084	5	1027	114	5.0	1-25	H372E YAH 376	280-525	603-912	956-1001
12	Aedes albopictus	AA017287	3322	ATG	TGA	3003	5	1000	112	4.9	1-23	H359E YAH 363	285-512	590-903	946-974
13	Aedes aegypti	AA012778	2957	ATG	TAA	2697	4	898	112	4.8	1-22	H359E YAH 363	285-512	590-898	-
14	Culex quinquefasciatus	CPIJ001048	3305	ATG	TAA	3036	5	1011	113	4.9	1-20	H299E YAH 303	285-512	590-912	866-925

Table 2. List of genomic sequences of APN gene of various non-mosquitos (Diptera)

Sr. no	Order	Family	Species	Accession No.	Length	ORF	Exons	Introns	AA	Mol. Wt.	Signal Peptide	Mature peptide	Start codon	Stop codon	Peptide sequence_M1	ER AP 1_C
1.	Diptera	Drosophilidae	<i>Drosophila ananassae</i>	FBtr0122736	3274	2886	5	4	961	108	1-20	21-961	ATG	TAG	298-510	589-890
			<i>Drosophila mojavensis</i>	FBgn0147011	3037	2859	4	3	952	107	1-20	22-952	ATG	TAA	291-505	585-902
			<i>Drosophila virilis</i>	FBgn0210169	3143	2850	4	3	949	109	1-22	23-949	ATG	TAG	288-485	582-902
			<i>Drosophila erecta</i>	FBgn0109703	3180	2862	4	3	953	106	1-24	25-953	ATG	TAG	294-508	587-888
			<i>Drosophila melanogaster</i>	FBgn0051233	3185	2859	4	3	952	106	1-25	26-952	ATG	TAA	293-507	586-904
			<i>Drosophila persimilis</i>	FBgn0161378	3092	2877	4	3	958	107	1-25	26-958	ATG	TAG	294-505	585-888
			<i>Drosophila grimshawi</i>	FBgn0126717	3348	2880	5	4	959	109	1-20	21-959	ATG	TAA	297-508	588-897
			<i>Drosophila pseudoobscura</i>	FBgn0076210	3089	2877	4	3	958	107	1-22	23-958	ATG	TAG	294-505	585-887
			<i>Drosophila yakuba</i>	FBgn0228109	3504	2883	5	4	960	108	1-22	23-960	ATG	TAA	299-510	591-888
			<i>Drosophila sechellia</i>	FBgn0181222	3575	2900	5	4	966	108	1-22	23-966	ATG	TAA	303-515	595-893
		<i>Drosophila simulans</i>	FBgn0192352	3566	2886	5	4	961	108	1-22	23-961	ATG	TAA	298-510	590-888	
		<i>Drosophila willistoni</i>	FBtr0243538	3065	2871	4	3	956	108	1-21	22-956	ATG	TAA	293-505	584-887	
		Muscoidae	<i>Musca domestica</i>	MDOA004623	8955	2346	5	4	781	89	-	-	GGC	TAA	106-330	409-705
		Psychodidae	<i>Stomoxys calcitrans</i>	SCAU003701	6181	2871	5	4	956	108	1-25	25-956	ATG	TAG	283-507	586-904
	<i>Lutzomyia longipalpis</i>	LLOJ009515	4700	2808	6	5	934	105	1-21	22-934	ATG	TGA	270-497	578-879		

mosquito APN. Furthermore, other insects belonging to Diptera also have more of the alanine and leucine amino acids (Table 5). In some non-mosquitoes like *Drosophila grimshawi* a significant decline in the num-

ber of alanine residues was noticed with an increase in arginine and serine residues. The Leucine residues are present in highest frequency in the amino acid composition of protein of each species (accounting for 9% of

Table 3. Nucleotide composition of APN gene of mosquitoes (Diptera)

Sno.	Species	A+T %			G+C%			AT	GC
1.	<i>Aedes aegypti</i>	47.0	61.4	46.2	53.2	38.5	53.6	51.5	48.5
2.	<i>Culex quinquefasciatus</i>	47.4	59.2	41.6	52.3	40.9	58.1	49.6	50.4
3.	<i>Anopheles darlingi</i>	44.3	56.1	30.4	56.0	43.9	69.9	43.4	56.6
4.	<i>Anopheles culicifacies</i>	45.0	55.4	29.6	55.1	44.9	70.1	43.4	56.7
5.	<i>Anopheles arabiensis</i>	44.5	55.9	30.9	55.4	43.8	68.8	44.0	56.0
6.	<i>Anopheles gambiae</i>	44.5	56.9	30.7	55.4	43.6	69.5	43.9	56.1
7.	<i>Anopheles merus</i>	44.5	56.0	32.3	55.6	43.6	68.1	44.3	55.7
8.	<i>Aedes albopictus</i>	46.9	58.3	40.8	53.3	41.1	59.6	48.6	51.3
9.	<i>Anopheles albimanus</i>	42.2	56.6	22.0	57.8	43.5	77.9	40.4	59.7
10.	<i>Anopheles atroparvus</i>	43.6	55.6	20.4	56.8	44.4	80.0	39.5	60.4
11.	<i>Anopheles farauti</i>	41.8	56.7	18.3	58.0	43.7	81.5	38.9	61.1
12.	<i>Anopheles maculatus</i>	44.4	56.7	33.0	56.0	43.0	67.0	44.6	55.3
13.	<i>Anopheles quadriannulatus</i>	44.6	55.8	31.4	55.6	43.9	68.1	44.2	55.8
14.	<i>Anopheles sinensis</i>	43.6	56.0	25.5	56.0	44.3	74.6	41.6	58.3
15.	<i>Anopheles stephensai</i>	43.1	55.2	23.0	56.4	45.3	77.5	40.3	59.8

Table 4. Nucleotide composition of APN gene of non-mosquitoes (Diptera)

Sno.	Species	A+T %			G+C%			AT	GC
1.	<i>Drosophila melanogaster</i>	46.6	61.5	30.9	53.4	38.4	68.8	46.5	53.6
2.	<i>Drosophila mojavensis</i>	44.3	60.6	34.9	55.9	38.1	65.1	46.5	53.4
3.	<i>Drosophila virilis</i>	45.9	60.7	38.9	53.8	39.2	61.5	48.5	51.5
4.	<i>Drosophila erecta</i>	45.6	60.8	25.0	53.9	39.8	74.8	44.0	56.0
5.	<i>Drosophila ananassae</i>	49.7	58.8	30.8	48.0	40.2	68.9	53.2	53.2
6.	<i>Drosophila persimilis</i>	48.1	59.3	24.8	52.2	51.7	75.3	56.1	56.1
7.	<i>Drosophila grimshawi</i>	61.3	47.9	50.1	39.0	40.7	49.5	46.7	46.7
8.	<i>Drosophila pseudoobscura</i>	48.4	59.7	25.0	52.0	42.2	75.2	55.9	55.9
9.	<i>Drosophila yakuba</i>	51.8	57.9	29.0	55.9	39.0	71.0	54.0	54.0
10.	<i>Drosophila sechellia</i>	45.3	60.6	34.9	48.9	42.7	65.5	53.4	53.4
11.	<i>Drosophila simulans</i>	50.3	56.9	32.9	49.0	-	67.1	47.1	52.5
12.	<i>Drosophila willistoni</i>	51.3	59.8	54.8	45.7	39.8	45.1	55.4	44.7
13.	<i>Musca domestica</i>	50.6	62.7	45.6	50.4	37.2	54.6	54.1	45.8
14.	<i>Stomoxys calcitrans</i>	54.4	60.3	40.3	49.4	38.8	59.4	50.5	49.6
15.	<i>Lutzomyia longipalpis</i>	51.0	60.8	53.4	49.4	39.6	46.7	54.7	45.2

total amino acids) whereas cysteine residues are least abundant (1%) in the protein of insects (Table 6).

Synonymous Codon Usage: Codon bias is the non-uniform use of synonymous codons which encode the same amino acid. Some codons are more frequently used than others in several organisms, particularly in highly expressed genes. The spectacular diversity of insects makes them a suitable candidate for analysing the codon usage bias. To understand the pattern of non-random usage of synonymous codons in the insects, RSCU of individual codons were compared between mosquitoes and non-mosquitoes groups of species. RSCU > 1 represents codons that are used more frequently than expected whereas < 1 represents codons which are used less frequently. The overall RSCU values for the 59 codons in *Anopheles* indicated that C and G occurred more frequently at the third codon position (as shown in Table 7). These are TTC, CTG, ATC, GTG, TCC, TCG, CCG, ACC, ACG, GCC, GCG, TAC, CAG, AAC, AAG, GAG, TGC, CGC and GGC. Among

these frequent codons, the two most frequently used codon having highest values are CTG/Leu and CCG/Pro and amino acids such as asparagine, lysine, glutamine, phenylalanine, and tyrosine are encoded by one of the preferential synonymous codon. *Ae. aegypti* and *Cx. quinquefasciatus* APN genes also exhibit high synonymous codon usage bias (Table 7). The number of frequently used codons and rarely used codons varies among species, for example there were three amino acids in *Ae. aegypti* were GAG is preferred over GAA for glutamic acid and CCG is preferred over CCA for proline. The APN gene of mosquitoes showed a high synonymous codon usage bias in comparison to other insects, preferentially using one or two synonymous codons (Table 7-8). Three codons (CGC/Arg, ACC/Thr and UCC/Ser) are used frequently in Diptera. Furthermore some codons are found that are used frequently in each species of Diptera order of insects (Table 7-8). Specific codon such as CUA/Leu and GGG/Gly are found that are avoided in each species of each order. Within

Table 5. Amino acids composition of APN in mosquitoes (Diptera)

S no	Species	Ala	Cys	Asp	Glu	Phe	Gly	His	Ile	Lys	Leu	Met	Asn	Pro	Gln	Arg	Ser	Thr	Val	Trp	Tyr
1.	<i>Aedes aegypti</i>	8.35	0.55	5.34	6.3	3.45	4.9	1.78	5.79	4.12	9.24	2.00	6.12	4.23	3.78	4.45	5.67	8.12	7.57	2.33	5.79
2.	<i>Culex quinquefasciatus</i>	9.59	0.59	5.24	6.42	3.65	4.94	2.07	6.13	3.36	9.69	2.07	6.13	3.65	3.06	4.45	6.23	9.59	6.33	1.87	4.84
3.	<i>Anopheles darlingi</i>	9.61	0.78	5.78	5.69	3.33	5.20	2.45	4.80	1.96	10.6	2.25	4.71	4.31	3.63	5.49	5.78	10.9	5.88	1.76	4.80
4.	<i>Anopheles culicifacies</i>	10.6	1.07	4.96	5.74	3.99	4.96	1.46	4.47	2.23	9.44	2.72	5.06	4.18	3.21	5.55	6.62	9.93	7.10	1.94	4.67
5.	<i>Anopheles arabiensis</i>	10.2	0.68	5.09	5.49	3.72	5.98	1.66	4.50	2.64	9.80	2.64	5.39	3.82	3.62	5.09	6.17	10.0	6.66	1.76	4.90
6.	<i>Anopheles gambiae</i>	10.2	0.68	5.09	5.58	3.72	5.98	1.76	4.50	2.64	10.0	2.64	5.29	3.72	3.62	4.90	6.17	10.0	6.56	1.76	4.80
7.	<i>Anopheles merus</i>	9.90	0.68	5.29	5.58	3.72	5.98	1.66	4.60	2.74	9.60	2.64	5.19	3.82	3.62	5.19	6.37	9.90	6.86	1.76	4.80
8.	<i>Aedes albopictus</i>	9	1	5.3	6	3.9	5	2	6	3	9	2	5.7	4	4	4	7	9	7	2	5
9.	<i>Anopheles albimanus</i>	10.5	0.59	5.51	5.91	3.25	5.32	2.26	4.63	2.56	10.1	2.16	4.23	4.03	3.94	5.32	5.91	9.95	6.79	1.77	5.12
10.	<i>Anopheles atroparvus</i>	10.8	0.78	5.36	5.46	3.60	6.24	1.75	5.26	2.04	9.65	2.34	4.97	3.90	3.31	5.36	5.56	10.0	7.12	1.75	4.58
11.	<i>Anopheles farauti</i>	10.8	0.58	5.65	5.36	4.09	4.87	1.95	4.39	1.85	9.75	2.24	4.68	4.09	3.41	5.75	5.56	10.2	8.19	1.75	4.68
12.	<i>Anopheles maculatus</i>	10.2	0.64	4.76	7.90	4.11	5.30	1.94	5.08	2.16	8.87	2.16	4.97	4.11	3.46	6.70	5.95	8.00	6.16	1.83	5.51
13.	<i>Anopheles quadriannulatus</i>	10	0.68	5.29	5.58	3.72	5.88	1.66	4.41	2.64	9.70	2.64	5.19	3.72	3.52	5.19	6.47	10.1	6.86	1.76	4.80
14.	<i>Anopheles sinensis</i>	9.90	0.58	4.75	6.40	3.39	5.82	1.45	4.27	2.33	10.1	2.03	5.24	4.07	3.00	5.43	5.53	11.1	7.76	1.84	4.75
15.	<i>Anopheles stephensi</i>	10.6	0.68	5.20	5.29	3.82	5.59	1.57	4.71	1.96	10.1	1.96	4.90	3.72	3.33	5.59	6.77	10.5	6.96	1.76	4.80

Table 6. Amino acids composition of APN in non-mosquito Diptera

S no	Species	Ala	Cys	Asp	Glu	Phe	Gly	His	Ile	Lys	Leu	Met	Asn	Pro	Gln	Arg	Ser	Thr	Val	Trp	Tyr
1.	<i>Drosophila melanogaster</i>	8.19	0.73	5.46	6.09	4.62	5.14	2.10	4.09	3.67	10.8	1.36	6.51	3.67	4.62	3.46	7.14	8.29	7.24	1.68	5.04
2.	<i>Drosophila mojavensis</i>	9.13	0.73	5.77	5.98	4.30	4.72	2.20	4.30	3.67	10.9	1.68	6.09	3.88	4.93	4.20	6.93	7.56	6.19	1.78	4.93
3.	<i>Drosophila virilis</i>	9.16	0.73	5.58	6.63	4.53	4.53	2.31	4.53	3.79	10.7	1.47	6.53	3.58	4.32	3.89	6.63	8.74	5.90	1.79	4.53
4.	<i>Drosophila erecta</i>	8.49	0.62	5.24	5.35	4.93	5.56	2.09	3.67	3.67	10.2	1.36	6.71	3.67	4.93	3.56	7.66	7.97	7.66	1.67	4.82
5.	<i>Drosophila ananassae</i>	7.59	0.72	5.61	5.20	4.26	4.47	2.08	5.09	2.70	10.8	1.14	6.76	3.64	4.16	4.05	9.78	8.42	5.82	2.28	5.30
6.	<i>Drosophila persimilis</i>	8.49	0.73	5.24	6.19	5.03	4.72	1.99	4.82	3.25	11.2	1.36	7.24	3.77	5.03	3.46	6.92	7.13	6.71	1.78	4.82
7.	<i>Drosophila grimshawi</i>	5.64	3.87	0.73	1.35	2.92	3.45	3.55	7.21	3.13	10.0	4.81	3.97	7.84	3.76	10.5	10.0	8.15	4.07	2.61	2.19
8.	<i>Drosophila pseudoobscura</i>	8.35	0.62	6.15	4.69	4.48	4.90	1.77	4.59	3.02	11.4	1.04	6.88	3.13	5.11	3.86	9.39	8.03	5.01	2.40	5.01
9.	<i>Drosophila yakuba</i>	7.60	0.63	5.63	4.68	4.27	4.68	1.88	5.31	3.02	10.7	1.04	6.56	3.13	3.43	4.38	10.1	9.47	5.52	2.18	5.72
10.	<i>Drosophila sechellia</i>	9.13	0.73	5.77	5.98	4.30	4.72	2.20	4.30	3.67	10.9	1.68	6.09	3.88	4.93	4.20	6.93	7.56	6.19	1.78	4.93
11.	<i>Drosophila simulans</i>	8.22	0.62	5.93	4.37	4.37	4.47	1.76	5.41	3.01	10.5	1.04	6.55	3.32	3.53	4.37	9.78	9.46	5.09	2.39	5.72
12.	<i>Drosophila willistoni</i>	8.05	0.83	5.54	6.17	4.70	4.39	2.19	5.33	3.45	9.20	1.15	6.06	3.24	4.81	3.66	8.47	8.99	5.96	2.19	5.54
13.	<i>Musca domestica</i>	7.55	0.76	5.63	5.12	3.84	4.73	2.43	5.63	5.12	8.57	2.30	6.91	3.58	4.99	3.58	8.19	6.65	5.76	2.17	6.40
14.	<i>Stomoxys calcitrans</i>	8.47	0.62	5.64	5.64	4.18	5.33	2.82	5.43	3.87	8.68	2.40	7.32	4.28	4.18	4.39	6.38	7.21	5.75	2.09	5.23
15.	<i>Lutzomyia longipalpis</i>	7.81	0.85	6.42	4.92	4.81	5.56	1.71	6.95	2.03	9.20	2.78	6.10	3.85	3.64	4.49	6.20	8.77	5.78	2.03	5.99

the Insect, the preference shifts accomplished by high bias are seen in mosquitoes of Diptera and show preference shifts in three amino acids phenylalanine, histidine and threonine.

Effect of base composition on codon bias: Because of differences in mutational bias, the percentage of GC content varies greatly among different species, even in the species from the same order. An earlier study shows that genes in the Hymenoptera genome are generally localized in GC poor regions whereas among dipteran genomes such bias is not evident [18]. To determine the association of GC biasness with codon bias among different species, we move to the non-directional codon bias measures effective number of codon (ENC), which depends on nucleotide composition of genes [13]. An ENC value varies from 20 to 61, when ENC=20 means that only one codon is used for each amino acid (extreme codon bias) and ENC =61 means all codons are equally likely to code the amino acid (no biased usage of codon). The dipterans and particularly the mosquitoes were found to have the highest codon usage bias with an average ENC value of 45 and also with the highest average GC content at third codon position; 77.9 (Table 9). The greater the extent of codon preference in a gene, the lesser is the corresponding ENC value. The ENC values range from 40 to 56 in mosquitoes. Lower ENC value means more bias (Table 9 10). The average for the non-mosquito species

was 47.4. It was found that there was a positive correlation between GC content and the degree of synonymous codon usage bias measured by ENC. The highest synonymous codon usage bias was found in *An. albimanus* APN with an ENC of 40.4 and GC3s is 78% (9-10). CAI is a directional measure of codon usage bias similar to relative codon bias score unlike ENC. Thus comparison between CAI and ENC provides a good quantitative assessment between nucleotide composition and codon bias selection. This gene shows increase in CAI value with decrease in ENC value in Diptera. CAI is used to calculate the level of gene expression. The average CAI value ranges from 0.5 to 0.7, a level close to 1.0, which might give a high expression level of genes according to the index definition. *Drosophila willistoni* is found to have lower codon biases with highest ENC value.

Table 7. Relative Synonymous Codon Usage of APN gene in mosquitoes (Diptera)

Amino Acid	Codons	Ae. aegypti	Cu. quinquefasciatus	An. darlingi	An. culicifacies	An. arabiensis	An. gambiae	An. merus	Ae. albopictus	An. albimanus	An. atroparvus	An. farauti	An. maculatus	An. quadriannulatus	An. sinensis	An. stephensi
Phe (F)	TTT	0.77	0.54	0.29	0.2	0.47	0.37	0.42	0.56	0.24	0.22	0.29	0.26	0.47	0.34	0.21
	TTC	1.23	1.46	1.71	1.8	1.53	1.63	1.58	1.44	1.76	1.78	1.71	1.74	1.53	1.66	1.79
Leu (L)	TTA	0.14	0.12	0.11	0.31	0.18	0.29	0.18	0.07	0.06	0.06	0.06	0.29	0.18	0.06	0.06
	TTG	1.16	1.35	1.21	0.74	0.84	0.82	0.73	1.4	0.93	0.91	0.78	0.88	0.67	1.2	0.58
	CTT	1.23	1.04	0.5	0.49	0.42	0.47	0.43	0.63	0.06	0.48	0.06	0.8	0.42	0.23	0.47
	CTC	0.8	0.86	0.94	0.74	1.02	0.99	0.98	1.12	1.34	0.97	1.38	0.88	0.97	1.03	1.05
	CTA	0.29	0.37	0.61	0.49	0.66	0.52	0.67	0.42	0.52	0.12	0.3	0.8	0.61	0.4	0.41
CTG	2.39	2.27	2.64	3.22	2.88	2.91	3	2.37	3.09	3.45	3.42	2.34	3.15	3.09	3.44	
Ile (I)	ATT	1.5	1.16	0.49	0.59	0.85	0.85	0.96	1.36	0.26	0.72	0.8	0.64	0.87	0.41	0.69
	ATC	1.21	1.74	2.27	1.96	1.89	1.89	1.79	1.42	2.74	2.11	2.13	1.98	1.93	2.39	2.19
	ATA	0.29	0.1	0.24	0.46	0.26	0.26	0.26	0.22	0	0.17	0.07	0.38	0.2	0.2	0.13
Met (M)	AUG	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Val (V)	GTT	1.35	1.5	0.53	0.22	0.59	0.66	0.74	1.08	0.64	0.55	0.43	0.56	0.57	0.8	0.34
	GTC	0.88	0.75	1.4	1.1	0.88	0.9	0.8	1.03	0.87	1.1	0.95	1.12	0.86	1.1	0.85
	GTA	0.47	0.63	0.33	1.1	0.47	0.48	0.46	0.59	0.41	0.38	0.38	0.91	0.51	0.6	0.51
	GTG	1.29	1.13	1.73	1.59	2.06	1.97	2	1.3	2.09	1.97	2.24	1.4	2.06	1.5	2.31
Ser (S)	TCT	0.47	1.24	0.71	0.44	0.48	0.38	0.46	0.79	0.1	0.11	0.11	0.22	0.36	0.21	0.35
	TCC	1.41	1.24	0.51	1.24	1.33	1.43	1.57	1.5	1	1.79	1.89	1.31	1.36	1.47	1.48
	TCA	0.35	0.38	0.31	0.44	0.29	0.19	0.09	0.71	0.2	0.11	0	0.44	0.18	0.21	0.17
	TCG	1.53	0.86	2.24	1.94	2	2	1.94	0.79	2.3	2.53	1.89	1.75	2.09	1.89	1.74
	AGT	1.88	0.86	1.02	0.79	1.05	1.05	1.02	1.06	0.7	0.32	0.42	1.53	1	0.84	0.96
	AGC	0.35	1.43	1.22	1.15	0.86	0.95	0.92	1.15	1.7	1.16	1.68	0.76	1	1.37	1.3
Pro (P)	CCT	0.95	0.32	0.36	0.19	0.41	0.42	0.41	0.76	0.29	0.4	0.1	0.53	0.42	0.29	0.32
	CCC	0.95	0.76	0.55	1.02	0.72	0.74	0.82	1.05	0.49	1	1.14	0.84	0.74	0.76	0.95
	CCA	1.37	1.3	0.64	0.28	1.03	0.95	1.03	1.05	0.59	0.3	0.19	0.74	0.95	0.57	0.42
	CCG	0.74	1.62	2.45	2.51	1.85	1.89	1.74	1.14	2.63	2.3	2.57	1.89	1.89	2.38	2.32
Thr (T)	ACT	0.93	0.66	0.5	0.47	0.55	0.66	0.59	0.75	0.4	0.54	0.27	0.43	0.65	0.52	0.41
	ACC	1.53	1.73	1.39	1.22	1.53	1.4	1.35	1.58	1.31	1.79	1.75	1.51	1.38	1.57	1.38
	ACA	0.6	0.41	0.57	0.39	0.27	0.27	0.32	0.4	0.36	0.23	0.27	0.22	0.35	0.28	0.07
	ACG	0.93	1.2	1.54	1.92	1.65	1.67	1.74	1.27	1.94	1.44	1.71	1.84	1.62	1.63	2.13
Ala (A)	GCT	1.07	1.03	0.69	0.59	0.76	0.72	0.79	0.72	0.49	0.29	0.29	0.59	0.75	0.55	0.29
	GCC	1.12	1.48	1.51	1.36	1.18	1.22	1.11	1.57	1.64	1.77	1.44	1.52	1.18	1.88	1.76
	GCA	1.07	0.78	0.82	0.77	0.95	0.88	0.99	1.11	0.34	0.36	0.4	0.72	0.98	0.59	0.62
	GCG	0.75	0.7	0.98	1.28	1.1	1.18	1.11	0.6	1.53	1.59	1.87	1.18	1.1	0.98	1.32
Tyr (Y)	TAT	0.58	0.73	0.37	0.58	0.44	0.45	0.45	0.72	0.35	0.3	0.25	0.59	0.49	0.37	0.24
	TAC	1.42	1.27	1.63	1.42	1.56	1.55	1.55	1.28	1.65	1.7	1.75	1.41	1.51	1.63	1.76
STOP	TAA(*)	3	3	0	3	3	3	3	0	3	3	3	0	3	3	3
	TAG(*)	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0
His (H)	CAT	1.25	0.86	0.64	1.2	1.18	1	1.18	0.7	0.7	0.56	0.5	1	1.18	0.4	1.25
	CAC	0.75	1.14	1.36	0.8	0.82	1	0.82	1.3	1.3	1.44	1.5	1	0.82	1.6	0.75
Gln (Q)	CAA	0.94	0.65	0.59	0.42	0.27	0.27	0.32	1.1	0.35	0.24	0.23	0.31	0.28	0.32	0.18
	CAG	1.06	1.35	1.41	1.58	1.73	1.73	1.68	0.9	1.65	1.76	1.77	1.69	1.72	1.68	1.82
Asn (N)	AAT	0.91	0.55	0.67	0.5	0.65	0.63	0.68	0.67	0.33	0.24	0.17	0.74	0.64	0.41	0.32
	AAC	1.09	1.45	1.33	1.5	1.35	1.37	1.32	1.33	1.67	1.76	1.83	1.26	1.36	1.59	1.68
Lys (K)	AAA	0.97	0.94	0.3	0.52	0.3	0.3	0.43	0.63	0.31	0.19	0.32	0.6	0.44	0.25	0.4
	AAG	1.03	1.06	1.7	1.48	1.7	1.7	1.57	1.38	1.69	1.81	1.68	1.4	1.56	1.75	1.6
Asp (D)	GAT	1.21	0.98	1.15	1.06	1.04	1	1	1.06	1.14	0.44	0.93	0.91	1	0.73	0.72
	GAC	0.79	1.02	0.85	0.94	0.96	1	1	0.94	0.86	1.56	1.07	1.09	1	1.27	1.28
Glu (E)	GAA	1.19	1.48	0.55	0.85	0.64	0.7	0.67	1.11	0.53	0.68	0.73	0.82	0.77	0.73	0.74
	GAG	0.81	0.52	1.45	1.15	1.36	1.3	1.33	0.89	1.47	1.32	1.27	1.18	1.23	1.27	1.26
Cys (C)	TGT	0.8	1	0.5	1.27	0.86	0.86	0.86	0.8	0.33	0	0.67	0.67	0.86	0.33	0.86
	TGC	1.2	1	1.5	0.73	1.14	1.14	1.14	1.2	1.67	2	1.33	1.33	1.14	1.67	1.14

Amino Acid	Codons	Ae. aegypti	Cu. quinquefasciatus	An. darlingi	An. culicifacies	An. arabiensis	An. gambiae	An. merus	Ae. albopictus	An. albimanus	An. atroparvus	An. farauti	An. maculatus	An. quadriannulatus	An. sinensis	An. stephensi
STOP	TGA(*)	0	0	0	0	0	0	0	3	0	0	0	3	0	0	0
Trp (W)	TGG	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Arg (R)	CGT	1.2	2.13	1.5	2	1.62	1.56	1.58	1.4	0.78	0.87	0.51	0.87	1.7	1.71	0.63
	CGC	1.35	1.33	2.68	2	2.42	2.4	2.49	1.12	2.56	2.84	2.24	1.94	2.38	2.14	2.74
	CGA	1.8	1.07	0.64	0.21	0.81	0.96	0.91	1.53	0.22	0.33	0.41	0.77	0.91	0.54	0.84
	CGG	0.6	0.4	0.75	1.26	0.69	0.72	0.57	0.7	2.11	1.31	2.34	0.87	0.57	0.96	1.16
	AGA	1.05	0.53	0.21	0.21	0.35	0.24	0.23	0.42	0.22	0.44	0.2	1.26	0.23	0.32	0.21
	AGG	0	0.53	0.21	0.32	0.12	0.12	0.23	0.84	0.11	0.22	0.31	0.29	0.23	0.32	0.42
Gly (G)	GGT	1	1.28	1.96	1.57	1.38	1.31	1.25	1.2	1.93	1.63	1.28	1.39	1.4	1.47	1.33
	GGC	1.18	0.64	0.98	1.18	1.44	1.57	1.57	0.8	1.26	1.5	1.6	1.63	1.33	1.13	1.61
	GGA	1.73	2.08	0.75	0.63	0.85	0.79	0.92	1.68	0.44	0.63	0.48	0.82	0.87	0.87	0.7
	GGG	0.09	0	0.3	0.63	0.33	0.33	0.26	0.32	0.37	0.25	0.64	0.16	0.4	0.53	0.35

Amino Acids Sequence Alignment: Multiple sequence alignment of APN protein sequences were performed separately to know the conservation patterns within an order. The complete sequences of amino acids of APN belonging to Diptera order have been analysed. Sequence analysis of APN indicates all are evolved from common ancestors. The amino acids sequence alignment of is shown in Fig.1 2. The areas of highly conserved regions for amino acid were clearly observed. From the MSA it is clear that residues GAMEN, HEXXH and NEGFA are highly conserved, showing that these conserved sites are present in almost all the aligned sequences. All these three motifs are found conserved in Anopheles at similar positions. These repeats were also found in other mosquito species and showed homology to other insects. Glycosylation sites are also found conserved in all mosquitoes. Four cysteine residues are also found conserved in all insects. GPI prediction program exhibited the presence of GPI anchor signal sequence at the C-terminus. Such a sequence was found to be identical with An. gambiae and An. stephensi. Furthermore analysis of the C-terminal part of all insect APNs revealed a high degree of conservation of tyrosine residues.

Phylogenetic Analysis: Coding regions of AcAPN1 were examined for Phylogenetic analysis to study the evolutionary patterns among mosquitoes. This analysis includes 15 selected mosquitoes for the APN1 gene. The evolutionary pattern on phylogenetic tree showed that Anopheles, Aedes and Culex APN1 gene may have originated from common ancestors and revealed close similarity to each other. The relationship among all

three genus of mosquito is more strikingly represented in two clades: Anopheles and Aedes/ Culex, this show Aedes and Culex shows close similarity than Anopheles. The evolutionary relationship of APN1 protein derived from different Anopheles was also evaluated, among this Anopheles group An. culicifacies found to be placed nearer to An. stephensi and An. gambiae, to these which is in agreement with the peptide sequences of these genera as all contains GAMEN catalytic domain (Fig. 3). The tree topology shows that the Anopheles APN1 shares orthologs from other mosquitoes such as Aedes and Culex. Phylogenetic tree based on APN protein of fifteen species of Diptera order of Non mosquitoes were analysed separately to know the evolutionary relationship of organisms within an order. Phylogenetic tree also supported the grouping of species into two major clades. Phylogenetic analysis of APN indicates that all are evolved from common ancestors (Fig. 3 4).

DISCUSSION

Conserved DNA sequences of APN gene have been retrieved from 30 mosquitoes and non-mosquito species from NCBI, GenBank etc and has been analysed with various bioinformatic tools. The APN gene sequences consist of a start site, ATG followed by TAA (in most species) as termination codon site. The nucleotide sequences of all APN insects were found to have different numbers and positions of exons. The ORF region of APN gene almost falls in between 2-3 kb. The present investigation highlights comparative studies on nucleotide composition and codon bias between

Table 10. Relative Synonymous Codon Usage (RSCU) of APN gene in non-mosquitoes (Diptera)

Amino Acid	Codons	<i>D. ananassae</i>	<i>D. mojavensis</i>	<i>D. melanogaster</i>	<i>D. virilis</i>	<i>D. erecta</i>	<i>D. persimilis</i>	<i>D. grimshawi</i>	<i>D. pseudoobscura</i>	<i>D. yakuba</i>	<i>D. sechellia</i>	<i>D. simulans</i>	<i>D. willistoni</i>	<i>Musca domestica</i>	<i>Stomoxys calcitrans</i>	<i>Lutzomyia longipalpis</i>
Phe (F)	TTT	0.59	0.54	0.59	1.02	0.64	0.54	1.14	0.47	0.54	0.54	0.71	0.84	0.53	0.75	0.67
	TTC	1.41	1.46	1.41	0.98	1.36	1.46	0.86	1.53	1.46	1.46	1.29	1.16	1.47	1.25	1.33
Leu (L)	TTA	0.12	0.12	0.12	0.24	0.06	0	1	0.07	0.06	0.18	0.12	0.41	0	0.14	0.42
	TTG	1.46	0.98	1.46	1.29	1.29	0.56	1.69	0.65	0.93	0.98	0.89	1.77	4.03	2.89	1.19
	CTT	0.7	0.4	0.7	0.12	0.43	0.5	0.56	0.38	0.47	0.4	0.65	0.75	0.45	0.58	1.33
	CTC	0.29	0.69	0.29	0.82	0.49	1.01	0.75	0.98	0.93	0.69	0.65	0.41	0.72	0.72	2.16
	CTA	0.64	0.75	0.64	0.71	0.12	0.17	0.94	0.44	0.35	0.75	0.59	1.43	0.18	0.43	0.35
	CTG	2.8	3.06	2.8	2.82	3.61	3.76	1.06	3.49	3.26	3.06	3.09	1.23	0.63	1.23	0.56
Ile (I)	ATT	1.69	1.17	1.69	1.4	1.29	0.91	0.78	0.82	0.94	1.17	0.75	1.29	1.5	1.38	1.43
	ATC	1.23	1.54	1.23	1.26	1.63	2.02	1.26	1.91	1.76	1.54	2.08	1.06	1.43	1.21	1.34
	ATA	0.08	0.29	0.08	0.35	0.09	0.07	0.96	0.27	0.29	0.29	0.17	0.65	0.07	0.4	0.23
Met (M)	AUG	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Val (V)	GTT	0.58	0.61	0.58	0.86	0.66	0.44	0.82	0.25	0.53	0.61	0.82	1.33	1.6	1.02	1.78
	GTC	1.04	1.02	1.04	1.29	1.15	1.81	0.82	1.58	1.28	1.02	1.06	0.84	1.33	1.09	0.52
	GTA	0.29	0.34	0.29	0.21	0.05	0.38	0.92	0.25	0.15	0.34	0.33	0.7	0.36	0.51	0.3
	GTG	2.09	2.03	2.09	1.64	2.14	1.38	1.44	1.92	2.04	2.03	1.8	1.12	0.71	1.38	1.41
Ser (S)	TCT	0.26	0.82	0.26	0.76	0.08	0.36	1.13	0.4	0.12	0.82	0.32	0.67	0.47	0.69	0.72
	TCC	2.12	1.18	2.12	1.43	2.3	2	0.81	1.67	1.92	1.18	1.98	1.19	1.88	1.57	0.83
	TCA	0.18	1	0.18	0.29	0.25	0.09	1.13	0.27	0.31	1	0.19	1.33	0.47	0.69	1.24
	TCG	0.79	0.55	0.79	1.05	0.58	1.36	0.75	1.33	1.42	0.55	1.15	0.89	0.75	1.28	0.41
	AGT	0.35	0.55	0.35	0.38	0.41	0	1.13	0.73	0.56	0.55	0.77	0.96	1.03	1.08	1.86
	AGC	2.29	1.91	2.29	2.1	2.38	2.18	1.06	1.6	1.67	1.91	1.6	0.96	1.41	0.69	0.93
Pro (P)	CCT	0.46	0.65	0.46	1.29	0.34	0.44	1.28	0.4	0.4	0.65	0.75	0.9	0.71	0.78	0.78
	CCC	2.29	1.73	2.29	1.53	2.4	2.56	0.59	2.4	1.73	1.73	1.88	1.29	2.14	2.63	1.56
	CCA	0.69	0.65	0.69	0.71	0.57	0.56	1.55	0.67	0.65	0.65	0.75	1.42	0.71	0.49	1.56
	CCG	0.57	0.97	0.57	0.47	0.69	0.44	0.59	1.2	1.2	0.97	0.63	0.39	0.43	0.11	0.11
Thr (T)	ACT	0.76	1.22	0.76	0.48	0.47	0.76	0.87	0.52	0.75	1.22	0.84	1.16	0.85	0.64	0.59
	ACC	2.13	1.44	2.13	1.64	2.37	2	0.41	1.82	1.85	1.44	1.63	0.88	2.31	2.26	0.73
	ACA	0.51	0.61	0.51	0.92	0.37	0.47	1.54	0.57	0.4	0.61	0.44	1.16	0.46	0.64	1.9
	ACG	0.61	0.72	0.61	0.96	0.79	0.76	1.18	1.09	1.01	0.72	1.1	0.79	0.38	0.46	0.78
Ala (A)	GCT	0.82	0.87	0.82	0.84	0.69	0.81	0.25	0.99	0.87	0.87	0.96	1.25	1.22	0.64	1.21
	GCC	2.56	1.98	2.56	1.93	2.57	2.57	0.52	2.85	2.19	1.98	2.13	1.56	2.31	2.91	1.15
	GCA	0.31	0.51	0.31	0.46	0.2	0.44	1.7	0.3	0.44	0.51	0.56	1.04	0.34	0.3	1.42
	GCG	0.31	0.64	0.31	0.55	0.4	0.3	0.96	0.6	0.38	0.64	0.35	0.16	0.14	0.15	0.22
Tyr (Y)	TAT	0.83	1.11	0.83	1.44	0.74	0.43	1.05	0.75	0.62	1.11	0.76	1.43	1.4	1	0.82
	TAC	1.17	0.89	1.17	0.56	1.26	1.57	0.95	1.25	1.38	0.89	1.24	0.57	0.6	1	1.18
STOP	TAA(*)	1	0	1	0	0	0	0.96	0	0	0	0	0	3	0	0
	TAG(*)	0	0	0	0	0	3	0.75	0	0	0	0	0	0	3	0
His (H)	CAT	0.8	0.76	0.8	1.45	0.9	0.53	0.82	0.71	1	0.76	0.59	1.43	0.95	0.81	1.38
	CAC	1.2	1.24	1.2	0.55	1.1	1.47	1.18	1.29	1	1.24	1.41	0.57	1.05	1.19	0.63
Gln (Q)	CAA	0.41	0.34	0.41	0.54	0.34	0.25	1.06	0.45	0.42	0.34	0.47	0.83	1.23	1.1	1
	CAG	1.59	1.66	1.59	1.46	1.66	1.75	0.94	1.55	1.58	1.66	1.53	1.17	0.77	0.9	1
Asn (N)	AAT	0.68	0.97	0.68	1.13	0.72	0.49	1.16	0.7	0.63	0.97	0.76	1.31	1.07	1.2	1.26
	AAC	1.32	1.03	1.32	0.87	1.28	1.51	0.84	1.3	1.37	1.03	1.24	0.69	0.93	0.8	0.74
Lys (K)	AAA	0.4	0.74	0.4	0.39	0.29	0.19	1.2	0.28	0.28	0.74	0.34	1.03	1.1	0.92	0.84
	AAG	1.6	1.26	1.6	1.61	1.71	1.81	0.8	1.72	1.72	1.26	1.66	0.97	0.9	1.08	1.16
Asp (D)	GAT	1.04	1.13	1.04	1.32	0.8	1	1.14	0.98	1.41	1.13	1.3	1.32	1.27	1.07	1.53
	GAC	0.96	0.87	0.96	0.68	1.2	1	0.86	1.02	0.59	0.87	0.7	0.68	0.73	0.93	0.47
Glu (E)	GAA	0.52	0.46	0.52	0.48	0.27	0.34	1.38	0.36	0.67	0.46	0.57	1.25	1.7	1.07	1.26
	GAG	1.48	1.54	1.48	1.52	1.73	1.66	0.62	1.64	1.33	1.54	1.43	0.75	0.3	0.93	0.74
Cys (C)	TGT	0.57	0.29	0.57	0.86	0.67	0.57	0.59	0.67	0.29	0.67	0.75	1.33	0.67	0.33	0.86
	TGC	1.43	1.71	1.43	1.14	1.33	1.43	1.41	2	1.33	1.71	1.33	1.25	0.67	1.33	1

30 species belonging to order Diptera of class Insecta. As synonymous is not uniform during the translational

Amino Acid	Codons	<i>D. ananassae</i>	<i>D. mojavensis</i>	<i>D. melanogaster</i>	<i>D. virilis</i>	<i>D. erecta</i>	<i>D. persimilis</i>	<i>D. grimshawi</i>	<i>D. pseudoobscura</i>	<i>D. yakuba</i>	<i>D. sechellia</i>	<i>D. simulans</i>	<i>D. willistoni</i>	<i>Musca domestica</i>	<i>Stomoxys calcitrans</i>	<i>Lutzomyia longipalpis</i>
STOP	TGA(*)	0	0	0	0	0	0	0	1.29	0	0	0	0	0	0	3
Trp (W)	TGG	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Arg (R)	CGT	1.64	1.65	1.64	1.62	1.24	1.27	0.48	0.81	1.14	1.65	1.57	1.89	3	2.86	1.29
	CGC	2.91	3.45	2.91	2.43	3	4	0.89	2.11	2.71	3.45	1.86	1.89	1.93	2.43	1.86
	CGA	0.36	0.15	0.36	0.97	0.53	0	0.83	1.14	0.14	0.15	0.57	0.51	0	0.14	0.71
	CGG	0.18	0.6	0.18	0.81	0.18	0.36	0.83	0.16	0.71	0.6	0.71	0.34	0.21	0.14	0.14
	AGA	0	0	0	0	0	0.18	1.25	0.49	0.43	0	0.57	0.86	0.64	0.14	1
	AGG	0.91	0.15	0.91	0.16	1.06	0.18	1.72	1.3	0.86	0.15	0.71	0.51	0.21	0.29	1
Gly (G)	GGT	1.39	1.24	1.39	1.86	0.83	0.8	0.97	1.02	1.16	1.24	1.02	1.71	2.05	1.73	1.08
	GGC	0.98	2.04	0.98	1.67	1.58	2.31	1.21	1.79	1.96	2.04	1.77	0.86	1.73	1.65	0.77
	GGA	1.47	0.53	1.47	0.19	1.51	0.71	1.09	0.68	0.89	0.53	1.21	1.43	0.22	0.47	1.69
	GGG	0.16	0.18	0.16	0.28	0.08	0.18	0.73	0.51	0	0.18	0	0	0	0.16	0.46

Table 12. Effective number of synonymous codons and GC content of APN gene in mosquitoes (Diptera)

Species	CAI	ENC	GC3s	GC %
<i>Culex quinquefasciatus</i>	0.749	52.1	58.1	50.4
<i>Aedes aegypti</i>	0.771	54.5	53.6	48.5
<i>Anopheles darlingi</i>	0.783	45.4	69.9	56.6
<i>Anopheles gambiae</i>	0.527	56.2	69.4	47.8
<i>Anopheles arabiensis</i>	0.734	47.9	68.8	56
<i>Anopheles merus</i>	0.661	48.2	68.1	55.8
<i>Anopheles culicifacies</i>	0.735	46.2	70	56.6
<i>Anopheles albimanus</i>	0.737	40.4	77.9	59.7
<i>Aedes albopictus</i>	0.705	55.4	59.5	51.4

Table 13. Effective number of synonymous codons and GC content of APN in non-mosquitoes (Diptera)

Species	CAI	ENC	GC3s	GC %
<i>Drosophila mojavensis</i>	0.714	45.8	65.5	53.5
<i>Drosophila ananassae</i>	0.801	40.4	74.8	55.9
<i>Drosophila virilis</i>	0.789	48.0	61.5	51.5
<i>Drosophila erecta</i>	0.790	40.8	74.8	56.0
<i>Drosophila sechellia</i>	0.796	42.1	70.8	54.2
<i>Drosophila melanogaster</i>	0.782	43.7	68.8	53.5
<i>Drosophila persimilis</i>	0.768	41.5	75.3	56.1
<i>Drosophila grimshawi</i>	0.661	55.2	52.2	47.4
<i>Drosophila pseudoobscura</i>	0.718	42.0	75.2	55.9
<i>Drosophila yakuba</i>	0.739	45.1	70.9	54.0
<i>Drosophila simulans</i>	0.748	48.0	67.1	52.9
<i>Drosophila willistoni</i>	0.808	55.2	45.1	44.7
<i>Musca domestica</i>	0.815	42.9	54.6	45.8
<i>Lutzomyia longipalpis</i>	0.777	52.7	46.7	49.5
<i>Stomoxys calcitrans</i>	0.748	45.7	59.4	45.3

of codons in protein coding genes in various insects' species. The overall RSCU values in mosquitoes indicated that C/G occurred more frequently at the 3rd codon position whereas in Hymenoptera, Lepidoptera and Hemiptera insect orders most frequent codon ends with A/T at the 3rd position (Sharma et al., 2014). In *Anopheles* and *Culex* species APN gene was found to be GC rich, whereas in *Aedes* AT richness was observed. Also for comparison, coding sequences of APN gene were taken from various insects to perform nucleotide composition analysis. All insect species were found to be AT rich except Diptera. The analysis shows that at 1st and 2nd codon positions, the A content was found higher than the T content except for the 3rd codon position. Dipterans APN gene was found to be GC rich and as compared to 1st and 2nd codon position high GC content was observed at 3rd codon position. These observations clearly indicate that the Diptera APN gene is a biased gene. The pattern of GC contents of the honey-bee genome and its relationship with codon usage was studied [19]. This showed that genes located in GC poor regions showed much larger deviation in both codon usage bias and amino acid bias than genes located in GC rich regions. The codon context pattern in the honey bee genome indicates that T and G combination occurs more frequently for adjacent 1st and 3rd positions of codon pairs than A and C combinations [20]. It thus suggested that such a pattern of codon context bias might have evolved in order to minimize the effect of nucleotide mutations coding regions. Except methionine and tryptophan, the APN1 amino acid composition revealed mosquito-specific amino acid usage. This study

process, the identification of the codon usage pattern is important to understand the translational selection

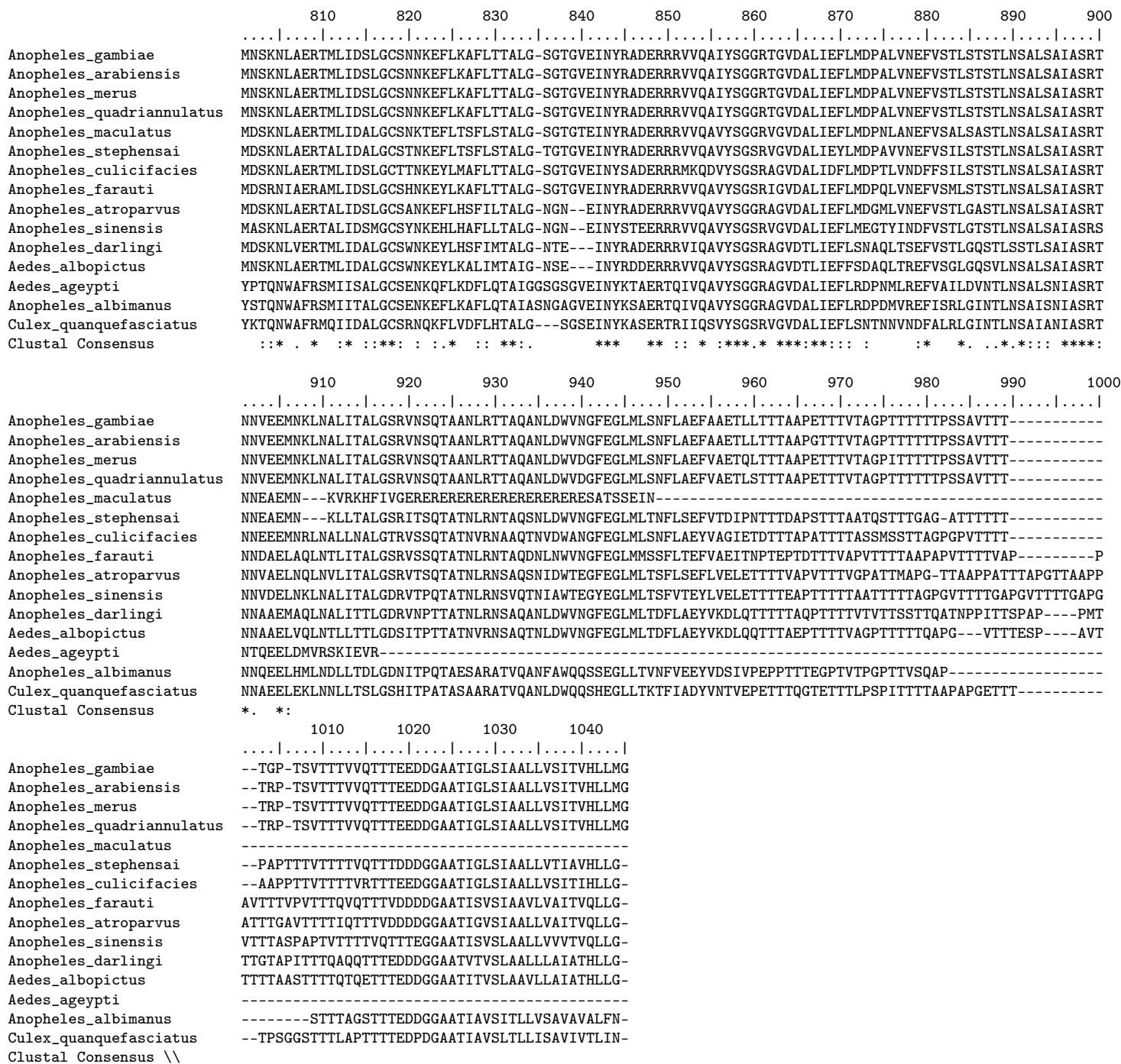


Figure 1. Amino acid sequence alignment of APN in mosquitoes (Diptera). Signal peptide was underlined at N terminal region. Yellow represents GAMEN motif, Blue represents HEXXH, Zn binding region, green represents NEGFA conserved regions, red represents conserved Cys residues, royal blue colour represents glycosylation sites and pink represents GPI anchored region at C terminal end.

veals the presence of thymine residues in high content [21]. The present study compares the codon usage patterns between different types of species from Diptera order of insects. As synonymous codon usage is not uniform during the translation process, the identification of the codon usage pattern is important to understand the translational selection of codons in protein coding genes among related species. A significant high synonymous codon usage bias was observed in mosquito APN

gene belonging to the order Diptera, by predominantly using one or two optimal synonymous codons over others. Similar results observed in non-mosquitoes species of Diptera order. In this study, the overall RSCU values for the 59 codons in mosquitoes indicated as C and G (C is more) occurs more frequently at the 3rd codon position. These results indicate that codon usage pattern in the mosquitoes is mostly contributed by compositional constraints. This main factor affects the

	10	20	30	40	50	60	70	80	90	100
Drosophila_persimilis
Drosophila_pseudoobscura
Drosophila_sechellia
Drosophila_simulans
Drosophila_yakuba
Drosophila_ananassae
Drosophila_willistoni
Drosophila_grimshawi
Drosophila_mojavensis
Drosophila_virilis
Drosophila_erecta
Drosophila_melanogaster
Stomoxys_calcitrans
Musca_domestica
Clustal Consensus
	110	120	130	140	150	160	170	180	190	200
Drosophila_persimilis
Drosophila_pseudoobscura
Drosophila_sechellia
Drosophila_simulans
Drosophila_yakuba
Drosophila_ananassae
Drosophila_willistoni
Drosophila_grimshawi
Drosophila_mojavensis
Drosophila_virilis
Drosophila_erecta
Drosophila_melanogaster
Stomoxys_calcitrans
Musca_domestica
Clustal Consensus
	310	320	330	340	350	360	370	380	390	400
Drosophila_persimilis
Drosophila_pseudoobscura
Drosophila_sechellia
Drosophila_simulans
Drosophila_yakuba
Drosophila_ananassae
Drosophila_willistoni
Drosophila_grimshawi
Drosophila_mojavensis
Drosophila_virilis
Drosophila_erecta
Drosophila_melanogaster
Stomoxys_calcitrans
Musca_domestica
Clustal Consensus
	410	420	430	440	450	460	470	480	490	500
Drosophila_persimilis
Drosophila_pseudoobscura
Drosophila_sechellia
Drosophila_simulans
Drosophila_yakuba
Drosophila_ananassae
Drosophila_willistoni
Drosophila_grimshawi
Drosophila_mojavensis
Drosophila_virilis
Drosophila_erecta
Drosophila_melanogaster
Stomoxys_calcitrans
Musca_domestica
Clustal Consensus

synonymous codon usage bias reported as in the case of *D. melanogaster* [22-23]. In *Drosophila* generally G and especially C are favoured at synonymous sites in biased genes. In *Anopheles*, the codon (CTG/Leu and CCG/Pro) are used more frequently. Amino acids like

phenylalanine, tyrosine, glutamine, asparagine, lysine and cysteine are almost solely encoded by one synonymous codon. The codon bias of *Aedes aegypti* and *Anopheles gambiae* mosquito's relative genome was analysed [24]. Genome wide analysis of

lation. In conclusion, codon bias is most prominent in highly expressed genes for translational competency and accuracy [29-30]. The results revealed the similar pattern was observed from other highly expressed genes [31]. So, higher codon biasness is found in APN1 gene among various mosquitoes and it is clear that APN1 is one of the highly expressed gene plays important in blood digestion and parasite invasion processes. APN amino acid sequence analysis shows the presence of high amino acid conservation among specific areas in all insects, all aligned confidently along their entire lengths. These include M1 Peptidase and ERAP1-like C-terminal domains. The zinc ions play an important role in the catalysis mechanism of metalloproteases like aminopeptidase. Multiple alignment results of different APN sequences showed the highest similarity between the Dipteran APN sequences [32]. In this study multiple alignments showed three major conserved sites in almost all the aligned sequences Where HEXXH motif is also conserved, which is responsible for peptidase activity [33-34]. The zinc metalloproteases like Aminopeptidase N among various phyla were shown to bear a signature sequence motif HEXXH where they bind the zinc atom in tetrahedral fashion. The highly conserved HEYAH amino acid residues are found in all insects [35]. Here the histidine residue acts as ligands of zinc ion where GLU plays a catalytic role. The GAMEN domain signature was also found in all insects and showed homology to each other. Many workers have determined hydrolysis and catalytic activities of APNs. Above said site GAMEN found as Bastatin binding site in APN of *E. coli* [36]. Third motif WWDNL found as a peptide binding site in human APN and it was found that this motif is also important in tumor angiogenesis and binding with peptides [37]. Also, these conserved sites are reported for Cry toxin protein binding region on APN [38]. The entire above said three motifs are found to be conserved in APN of *M. sexta* and *H. armigera* the similar sites were predicted by Conseq and Consurf servers [39]. APN protein of all the insects encoding amino acids sequence within 900-1000 amino acids and weights of APN protein was found in between 90 to 100 kDa. Another important finding is that APN follow secretory pathway, as amino terminal is encoded by signal sequences. Moreover, in the present investigation, GPI prediction program exhibited the presence of GPI anchor signal sequence at the C-terminus. Such sequence was found to be more conserved in dipterans and lepidopterans. Besides, the amino acid sequence also contained four Cysteine residues. Cys residues are important in the structural integrity of proteins by forming disulphide bridges, which in turn helps to keep the molecules intact and to maintain the conformation of

the active sites. These residues help in enhancing the stability of the proteins [40]. These residues are highly conserved among APN molecules of higher mammals (rat, rabbit, pig and human), was detected [41]. The post translational modification i.e. phosphorylation was found at serine, threonine and tyrosine residues conferring protection from proteolytic degradation. Protein-protein interaction results show that APN protein connected to glutathione synthase may involve in metabolic pathways for metabolism of Glutathione. Glutathione makes mosquito insecticide resistance, so disrupting of APN protein or any other protein, this protein network may block the whole metabolic pathway of Glutathione [42]. Amino acid sequence analysis of the Dipteran APN gene shows that they all can be aligned confidently along their entire lengths. Specific areas of relatively high amino acid conservation were observed. The ORF's range among all APN was found from 2 to 3.5 kb, encoding a 900-1000 amino acids sequence. All APN protein molecular weights were found to be above 90 kDa. Three important domains namely Aminopeptidase N like, M1 Peptidase and ERAP1-like C-terminal domain were identified in all APNs. All APNs are found to have four conserved Cysteine residues. Signal peptide sequence of 15-30 amino acids was present at N terminal end and GPI anchored region (hydrophobic amino acid residues) at C terminal end of APN protein. The APN analysis revealed the three conserved regions, gluzicin APN motif GAMEN, second one is Zinc-binding/ gluzicin motif HEXXH, which is a part of a typical catalytic site for the majority zinc dependent metallopeptidase and is required for enzymatic function, the third one is zinc binding ligand which is conserved in the sequence motif NEXFA. Highly conserved region is present in domain I, about 64 amino acid residues believed to be important for Cry 1 toxin binding. The Phylogram was constructed using the mosquitoes APN sequences and similar sequences of non-mosquitoes origin retrieved from the NCBI database. Undoubtedly, the ancestry species of the gene should be more closely related to sequences from a similar genus. In the present investigations the *An. gambiae* and *An. stephensi* seems to be the closest relatives of the *An. culicifacies* [43]. Out of the non-mosquitoes species, with no surprise are closely related to each other as they are the latest to diversify on the path of the evolutionary journey. Phylogenetic tree based on APN protein of non-mosquitoes were analysed separately to know the evolutionary relationship of organisms. Phylogenetic analysis of APN indicates that all are evolved from common ancestors. Phylogenetic tree also supported the grouping of each order of non-mosquitoes into two major clades. In Particular,

in Diptera two major clades belonged to Anopheline and Culicinae. The phylogenetic relationship followed by aligning the amino acid sequences from various insect species belonging to six distinct orders on one scale showed that the Lepidoptera and Diptera APN sequences situated into a single cluster [44]. The accumulation of similar sequences from other insects species reveal closer relative of the mosquitoes. Coleoptera and Hymenoptera were found in another single cluster. However, Hemiptera are situated in separate clades.

Conclusion

In silico analysis of the APN gene among six orders of class Insecta was also carried to understand more about APN genes and their evolution. A comparative sequence analysis was carried out to gain a better understanding of the evolution of APN gene. The conservation of residues was considerable and widespread throughout the coding region. However, the high level of divergence was observed in the size and positions of introns. This could be ascribed to the presence of repetitive DNA elements. Great sequence and structural diversity suggests that APN genes are highly evolving with selective functional constraints. APN sequences were found that contain conserved HEXXH and GAMEN signature sequences in multiple APN orthologs. The APN gene belonging to different orders was found to be AT rich except Diptera. Dipterans APN gene was found to be GC rich and at third codon position high GC content was observed. The presence of high GC content in Diptera clearly indicates that dipteran APN gene is a biased gene. The Leucine residues are present in highest frequency in the amino acid composition of protein of each species, accounting for 9% of total amino acids and cysteine residues are least abundant, 1% in the APN protein of insects. A significant high synonymous codon usage bias has been shown by mosquito APN genes belonging to the order Diptera (preferentially using one or two synonymous codons) as compared to other species.

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